CONTROLLING *Salmonella* IN POULTRY USING BACTERIOPHAGES

A Thesis

by

ANA GABRIELA SANCHEZ PENA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2012

Major Subject: Food Science and Technology

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Approved by:

Chair of Committee,	Christine Z. Alvarado
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ABSTRACT

Controlling *Salmonella* in Poultry Using

Bacteriophages. (August 2012)

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Chair of Advisory Committee: Dr. Christine Z. Alvarado

Public health concerns associated with high prevalence of foodborne salmonellosis, the emergence of antibiotic-resistant organisms and the identification of poultry meat and products as one of the most common sources of *Salmonella* support the need for new pathogen control strategies in the poultry industry. Scientific research has focused on the use of bacteriophages as therapeutic agents for humans and animals; however, limited studies have been conducted on bacteriophage application on food safety, especially on poultry. Therefore, the objective of this study was to evaluate the phage density and exposure time required to reduce *Salmonella* load on experimentally inoculated chicken meat.

In Experiment 1, serovars of *Salmonella* were tested for antimicrobial susceptibility and rifampicin-resistant isolates were generated. Cocktails of the serovars Enteritidis, Kentucky and Typhimurium (EKT), and Hadar and Heidelberg (HH), were inoculated on chicken breast samples to a target of 10^4 CFU/g. A mixture of three lytic bacteriophages, active against multiple *Salmonella* serovars was applied to chicken samples. A total of 84 samples (25 ±2 g) per each cocktail were distributed among a

negative control, *Salmonella*-inoculated positive control, *Salmonella*-inoculated samples treated with the phage mixture at differing titers (10^5, 10^6, 10^7, 10^8, and 10^9 PFU/ml) with two identical samples at 0, 15, 30, 60, 120, 360 min at 4°C. Experiment 2 evaluated nalidixic acid-resistant *Salmonella* Typhimurium among negative control, *Salmonella*-inoculated control (positive control), *Salmonella* with two phage titers (10^5 and 10^9 PFU/ml) at 0, 30, 60 and 120 min at 25°C and 4°C.

Results showed differences in means for *Salmonella* cocktail EKT ranged from 0.1 to 0.7 $\log_{10}$ CFU/g with 0.7 $\log_{10}$ for 10^8 PFU/ml, 30 min, 4°C. For *Salmonella* cocktail HH, reductions ranged from 0.1 to 0.4 $\log_{10}$ CFU/g with 0.4 $\log_{10}$ on samples treated with 10^8 PFU/ml, 120 min, 4°C. For the Experiment 2, a higher phage concentration (10^9 PFU/ml) at 120 min post-inoculation storage at 25°C was required to yield a 0.9 $\log_{10}$ difference in means. These findings showed that higher concentrations of bacteriophage were more effective controlling *Salmonella* than lower ones at both temperatures. In addition, temperature, time and bacterial attachment may influence phage efficacy.

DEDICATION

To my Parents, Ana María and José, and my
siblings Dani, David and Mari for their
love and support despite the distance.

To María and Kurt, for their love,
support and guidance.

To Walker, for his unconditional love,
moral support, patience and encouragement
throughout these past years.

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Finally, thanks to my soon to be husband Walker McClellan and my family for their encouragement, patience and love throughout the progression of my studies.

NOMENCLATURE

BG	Brilliant Green
CFU	Colony-forming unit
DMF	N,N-Dimethylformamide
g	Gram
h	Hours
MIC	Minimum inhibitory concentration
min	Minutes
ml	Milliliter
NA	Nalidixic Acid
L	Liter
log	Logarithmic (Base 10)
LUB	Luria Bertani Broth
PFU	Plaque forming unit
PW	Peptone water (0.1% w/v)
RIF	Rifampicin
rpm	Revolutions per minute
RTE	Ready-to-Eat
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth

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1. INTRODUCTION

Salmonella is one of the most commonly occurring foodborne pathogens in the United States. This microorganism has been estimated to present 1.2 million of salmonellosis cases per year, with approximately 42,000 laboratory-confirmed cases per year reported to the U.S. Centers of Disease Control and Prevention (CDC, 2012c). The actual number of infections is estimated at twenty-nine or more times the reported number due to non-reported or non–diagnosed milder cases (CDC, 2012c). This illness is transmitted to humans through contaminated food. Poultry meat and poultry products are common food sources of *Salmonella*.

Antibiotics have been widely utilized in farm environments as therapeutic agents and growth promoters in animal production (Joerger, 2003). However, current restrictions on the use of these products as growth promoters, and the development of antibiotic-resistant organisms, have supported the need for new pathogen control strategies to combat infectious illnesses (Mahony et al., 2011). Bacteriophage treatments have re-emerged as an innovative approach for pathogen control and an alternative to the use of antibiotics.

Bacteriophages, known also as phages, are naturally occurring viruses that infect bacterial cells. According to Sulakvelidze (2011), phages are the oldest and most ubiquitous organism on the Earth. Two types of bacteriophages can be found and both differ in their replication cycles. Virulent (also referred to as lytic) phages infect a

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This thesis follows the style of Journal of Poultry Science.

specific host, rapidly replicate and release mature phages by lysis. Temperate phages integrate their DNA with the one from their host and may transfer integrated fragments of bacterial DNA into another host bacterium (Sulakvelidze, 2011). Lytic (virulent) phages are favored for phage therapy due to the relatively fast bacterial destruction (Joerger, 2003) and inability for DNA transduction (Sulakvelidze, 2011).

Current research has focused on the use of bacteriophages as therapeutic agents on pre-harvest interventions on cattle and sheep for reduction of *Escherichia coli* O157:H7 (Niu et al., 2009; Sheng et al., 2006; Callaway et al., 2008), biocontrol of *Salmonella* on fresh-cut fruit (Leverentz et al., 2001), control of *Staphylococcus aureus* in pasteurized milk (Garcia et al., 2010), and prevention of *Salmonella* (Berchieri et al., 1991; Fiorentin et al., 2005a; Toro et al., 2005; Andreatti Filho et al., 2007; Atterbury et al., 2007; Borie et al., 2008; Ricci and Piddock, 2010) and prevention of *Campylobacter jejuni* (Carrillo et al., 2005; Wagenaar et al., 2005; Atterbury et al., 2007) colonization in poultry. Additional studies have been conducted on food products such as raw salmon fillets, catfish fillets and ready-to-eat (RTE) food treated against *Listeria monocytogenes,* control of *E. coli* O157:H7 on beef, fresh-cut honeydew treated against *L. monocytogenes* and *Salmonella* (Leverentz et al., 2001; Leverentz et al., 2004; O'Flynn et al., 2004; Guenther et al., 2009; Soni and Nannapaneni, 2010, Soni et al., 2010).

Research on bacteriophage application to control *Salmonella* in the post-harvest level of poultry production is limited. Higgins et al. (2005) and Bielke et al. (2007) found significant reduction on *Salmonella* recovery on their studies on broiler carcasses

after bacteriophage treatment. Goode et al. (2003) and Fiorentin et al. (2005b) reported reduction of experimentally contaminated *Salmonella* on chicken skin. Suppression of *Salmonella* growth in chicken frankfurters also has been studied (Whichard et al., 2003). The purpose of this research is to evaluate the phage density and exposure time required to reduce *Salmonella* load on experimentally inoculated chicken meat.

2. LITERATURE REVIEW

2.1 *Salmonella* spp.

2.1.1 Characteristics of the microorganism

Salmonella is one of the most common foodborne pathogens in the United States. *Salmonella* spp. are Gram-negative, non-sporing rod-shaped bacteria which belongs to the *Enterobacteriaceae* family. Members of this genus are facultative anaerobes, motile by peritrichous flagella and grow optimally at 37°C (D'Aoust and Maurer, 2007). They generally catabolize glucose and other monosaccharides, utilize amino acids as sole source of nitrogen, and are able to metabolize nutrients by both respiratory and fermentative pathways (Jay et al., 2005; D'Aoust and Maurer, 2007). Although these are general features for this organism, there are some variants that are non-motile due to lack of flagella. As well, some variants utilize lactose and/or sucrose, and are able to grow at extreme range of temperatures. For example, two mutants of mesophilic *Salmonella* Typhimurium were capable to growth at elevated temperatures, one at 48°C and another at 54°C as a result of extended exposure to these thermal stress conditions (Droffner and Yamamoto, 1992). In addition, there has been evidence that this same serovar was able to growth at a minimum temperature of 2°C in minced beef (Catsaras and Grebot, 1984, as cited by D'Aoust and Maurer, 2007) and minced chicken (Baker et al., 1986) which raise concerns on the safety of food during cold storage.

All the variety of salmonellae has been classified in two species, *S. enterica*, the type species, and *S. bongori*. *S. enterica* is divided in six subspecies, *S. enterica* subsp.

enterica, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica* (Brenner et al., 2000; Grimont and Weill, 2007), differentiated on the basis of biochemical and genomic features (D'Aoust and Maurer, 2007). Guibourdenche et al. (2010) reported that there are a total of 2,610 existing serovars distributed between these two species as of 2007, but most are in the *S. enterica* group. Overall, the most common serotypes related to *Salmonella* infections in 2010 were Enteritidis (22%), Newport (14%) and Typhimurium (13%) (CDC, 2011c).

2.1.2 Salmonellosis

Salmonella infections can be the cause of several human clinical conditions (D'Aoust and Maurer, 2007), such as enteric (typhoid) fever, and milder and severe cases of enterocolitis by non-typhoid *Salmonella* serovars. Non-typhoidal infections represent the most prevalent clinical cases of human salmonellosis, and are characterized by nausea, fever, abdominal pain, vomiting, chills and diarrhea. Symptoms occur 12 to 72 hours after consumption of food contaminated with *Salmonella* (Finstad et al., 2012). Milder cases of enterocolitis can show improvement after receiving fluids and electrolytes and do not require further treatment (D'Aoust and Maurer, 2007). Severe cases in which the infection can spread to the blood stream or results in extra-intestinal infections require hospitalization and medical treatment with antibiotics.

According to Jay et al. (2005), an oral dose of 10^7 to 10^9 CFU/g are the number of cells generally necessary to cause salmonellosis. However, lower numbers of cells

have also been related to *Salmonella* infections. The infectious dose required to cause salmonellosis also varies with the patient age and health, and with the composition of the food vehicle (Finstad et al., 2012). D'Aoust and Maurer (2007) indicated that low infectious doses are associated with contamination of high fat foods such as chocolate (cocoa butter), cheese (milk fat), and meat (animal fat). The chemical food composition may also be a determinant factor of salmonellosis. Authors such as D'Aoust (1977) and D'Aoust and Maurer (2007) have suggested that high fat content food would protect *Salmonella* cells from the acidic environment of the stomach, allowing them to colonize the lower portion of the small intestine. In addition, some studies (Glynn and Bradley, 1992; Mintz et al., 1994; Rejnmark et al., 1997) have reported a dose-severity relationship in infections with *Salmonella* isolates such as *S.* Enteritidis, *S.* Typhimurium, *S.* Infantis, *S.* Newport and *S.* Thompson.

Children, the elderly and persons with weakened immune systems are highly susceptible to *Salmonella* infections, and can present severe conditions (Bryan and Doyle, 1995; USDA-FSIS, 1996). Children less than 5 years old have been reported with the highest incidence of *Salmonella* infection in 2010, accounting for 69.5 laboratory-confirmed cases per 100,000 children (CDC, 2011c).

2.1.3 *Salmonella*: Reservoirs and trends

This bacterium is widespread in nature and is generally transmitted to humans through contaminated food. Its primary reservoir is the intestinal tract of animals (Antunes et al., 2003) and it is acquired from feed or feed ingredients, water and their

animate or inanimate environment (Bryan, 1980; Bryan and Doyle, 1995). Animals can carry *Salmonella* on their feet, skin, feathers, and hair contaminated with fecal material (Bryan, 1980). Beef, pork, poultry, eggs, and milk are the major sources of human salmonellosis (Gomez et al., 1997). In addition, *Salmonella* in raw poultry is an important cause of human salmonellosis (Mead et al., 2010) with 22.3% of human cases of *Salmonella* attributable to consumption of poultry products according to CDC's outbreak data for the period 1990 - 2006 (USDA-FSIS, 2008).

Every year, *Salmonella* accounts for approximately 1 million infections per year in the U.S. According to Food Disease Active Surveillance Network (FoodNet) data, 8,256 infections (17.6 illnesses per 100,000 persons) laboratory confirmed cases were reported in the 2010 (CDC, 2011c). This report also mentioned *Salmonella* as the most common cause of hospitalizations (2,290) and deaths (29), during 2010 (CDC, 2011c). FoodNet, which tracks food safety trends in the U.S., also reported a reduction on the incidence of several foodborne infections during the last 15 years: *Escherichia coli* O157:H7 (44% decrease), *Yersinia* (52% decrease), *Shigella* (57% decrease), *Listeria* (38% decrease) and *Campylobacter* (27% decrease); however, the incidence of *Salmonella* infections has increased (10% increase) in 2010, compared to 2006-2008 (CDC, 2011c).

2.1.4 Foodborne outbreaks associated with *Salmonella* spp.

The journal Morbidity and Mortality Weekly Report (MMWR) reported in the Surveillance for Foodborne Disease Outbreaks-U.S. that *Salmonella* was the second

most common cause of foodborne disease outbreaks (laboratory-confirmed outbreaks) during 2008, and was responsible for 23% of outbreaks, 31% of illnesses and 62% of hospitalizations (CDC, 2011b). This report also indicated poultry as the most common commodity related to *Salmonella* outbreaks. Nine multistate outbreaks of *Salmonella* were related to cantaloupe, ground white pepper, jalapeno and serrano peppers, cereal, ground turkey, and peanut butter and peanut paste. During 2010, a national outbreak of *Salmonella* infections caused by contamination of shell eggs led to a massive recall of these products (CDC, 2011c). In addition, CDC (2011b) indicated *Salmonella* in poultry as the pathogen-commodity pair responsible for the most outbreaks associated with *Salmonella*. *Salmonella* Enteritidis was the most common serotype of *Salmonella* outbreaks in 2010, representing the 27% of the 108 *Salmonella* outbreaks with a serotype reported (CDC, 2011b).

For the period 1998-2002, CDC (2006) reported “bare-handed contact by handler/worker/preparer” as the most common contamination factor contributing to foodborne disease outbreaks. For outbreaks caused by *Salmonella*, this report indicated that “raw product/ingredient contaminated by pathogens from animal or environment” and “cross-contamination from raw ingredient of animal origin,” were the main factors of contamination of food, whereas “allowing foods to remain at room or warm outdoor temperature for several hours,” and “insufficient time and/or temperature during initial cooking/heat processing,” are the primary factors for proliferation and survival of *Salmonella*, respectively. In addition, restaurants and private residences are the most

common reported places where food was eaten and resulted in *Salmonella* outbreaks (CDC, 2006).

2.1.5 Antibiotic resistance in *Salmonella* spp.

Antibiotics have been widely utilized on the farm environment as therapeutic agents and growth promoters in animal production (Joerger, 2003). A major worldwide public health concern is the emergence of antibiotic-resistant foodborne pathogens, as the resistant pathogens can be transmitted to humans through the food (Witte, 1998; White et al., 2002). The wide use of antibiotics in animals has resulted in non-typhoid resistant *Salmonella* serovars (Witte, 1998). Manie et al.(1998) and Antunes et al. (2003) have indicated high resistance of *Salmonella* spp. to one or more antimicrobial agents on poultry products, and this antibiotic-resistant spectrum is still increasing (Mor-mur and Yuste, 2010).

The National Antimicrobial Resistance Monitoring System (NARMS) is an initiative of the CDC in cooperation with the U.S. Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA) to monitor antimicrobial resistance among enteric bacteria isolated from humans. According to CDC (2012a), *S.* Enteritidis (55.1%) was the most common serovar resistant to nalidixic acid whereas *S.* Newport (31.4%) was the most common among the ceftriaxone-resistant non-typhoidal *Salmonella* isolates. Nalidixic acid is an elementary quinolone, and resistance to this antimicrobial agent correlates with a reduced susceptibility to ciprofloxacin, which with ceftriaxone and trimethoprim-sulfamethoxazole is a first-line antimicrobial for

salmonellosis treatment (CDC, 2012a; CDC, 2012b). This acquired or cross-resistance to antimicrobials is of public concern as treatment failure is possible due resistance microorganisms with an increase of the morbidity, mortality and costs related to the disease (Helmuth, 2000).

2.2 Poultry processing and *Salmonella* spp.

2.2.1 *Salmonella* in poultry production and processing

Poultry contamination has the potential to cause human infections through the transmission of *Salmonella* (Kimura et al., 2004; USDA-FSIS, 2011). Indeed, the prevalence of *Salmonella* in fowl and poultry products has a major influence on the risk for acquiring this human illness (Bryan and Doyle, 1995). At the farm, intestinal colonization and contamination of the body parts occurs in poultry, and is favored by the close proximity of birds due to the intensive raising operations (Bryan and Doyle, 1995). Contamination of feed, especially un-pelleted feed, is possible as it can contain raw ingredients mixed with heat-treated products. Litter and soil can become contaminated after fecal shedding and *Salmonella* present in the feces can survive in these environments for a few days (Bryan and Doyle, 1995). The exterior surface of the bird, especially feathers and skin, are another source of contamination in poultry plants (Molina, 2007; Corry and Atabay, 2001). Transportation of birds from farm to processing plants may also allow contamination to spread among birds.

Several steps during processing can increase microbial recovery or spread contamination. Scalding allows skin follicles to open for feather removal, remaining

open until chilling when follicles contract retaining microorganisms (Bryan and Doyle, 1995). Berrang (2001) reported that fecal material can escape the carcass by the cloaca (intestinal leakage) after defeathering, contaminating other carcasses. Chilling of the birds by immersion can also spread bacteria from contaminated to previously non-contaminated carcasses (Molina, 2007).

2.2.2 Bacterial attachment on meat surfaces

Molina (2007) stated that significant bacterial attachment and accumulation takes place during poultry processing. Some factors can influence bacterial attachment: cell surface charge, hydrophobicity, and cell surface structures (Dickinson and Koohmaraie, 1989). Bacterial cell structures such as fimbriae, flagella and pili were found to not be critical in attachment of *Salmonella* to poultry skin (Lillard, 1986). Benedict et al. (1991) showed specific attachment of *Salmonella* cells to the endomysial reticulin fibrils (collagen) rather than to muscle fibers of poultry. This was also confirmed by Sanderson et al. (1991) and Thomas and McMeekin (1981), who reported that *Salmonella* spp. attached primarily to collagen in poultry fascia, especially glycosaminoglycans that surround the collagen fibrils after extended immersion in water. In addition, changes in micro-topography in the muscle fascia may also be necessary for bacterial adhesion. Formation of deep ridges in the muscle during immersion resulted in expansion of the collagen (Thomas and McMeekin, 1981), which may make possible entrapment of bacteria in crevices on tissue surfaces having a barrier effect against antimicrobials (Lillard, 1988).

2.2.3 Government initiatives for the reduction of *Salmonella* spp.

Efforts for the control of *Salmonella* have been implemented by the U.S. Department of Agriculture Food Safety and Inspection Service. The Pathogen Reduction; Hazard Analysis and Critical Control Point (HACCP) systems Final Rule established the requirements applicable to meat and poultry establishment designed to reduce the prevalence and incidence of foodborne illnesses including *Salmonella* (UDSA-FSIS, 1996). *Salmonella* baseline results from studies conducted between 1997 and 2000 have become available to assist inspected establishment in assessing their processes (USDA-FSIS, 2005). In the 2010, FSIS released the third edition of the compliance guideline for controlling *Salmonella* and *Campylobacter* in poultry (USDA-FSIS, 2010), with recommendations for the industry to meet FSIS expectations with regard to food safety hazards. In addition, new performance standards for *Salmonella* in young chicken and turkey slaughter establishments, effective July 2011, have reduced the acceptable limit of *Salmonella* contamination at processing plants from 20% to 7.5% of carcasses (USDA-FSIS, 2011).

2.2.4 Pathogen interventions in the poultry industry

As the HACCP final rule went effective, fulfillment with zero tolerance for visible fecal contamination on carcasses and microbiological performance criteria was required for the meat and poultry industry. Interventions targeting *Salmonella* may also be beneficial in reducing the presence of other enteric pathogens (USDA-FSIS, 1996).

Methods that include the use of antimicrobial agents in rinses and spray washes for reduction of pathogen load have also been implemented.

According to Stopforth et al. (2007), major intervention strategies applied to the poultry industry can be divided in four categories: (i) scalding, where carcasses are submerged in a bath containing hot water ranging from 42°C (107.6°F) to 60°C (140°F); (ii) rinses/spray washes, using application of antimicrobial solutions post-picking, post-evisceration, pre- and post-chilling; (iii) on-line reprocessing, deluging and/or spraying of antimicrobial solutions to ensure that visible contamination is removed from carcasses; and (iv) carcass chilling, where carcasses are chilled by immersion in a cold bath containing antimicrobial solutions. As stated by Narendran (2003), antimicrobial agents must be non-toxic, should not affect the sensory attributes, be affordable and be easy to apply. In its Directive 7120.1 Rev 11, Food Safety and Inspection Service (FSIS) identified food grade chemical and biological antimicrobial agents approved for use in meat, poultry and egg products (USDA-FSIS, 2012). Common chemical agents used for decontamination of meat and poultry carcasses are chlorine or chlorine dioxide, acidified sodium chlorite, ozonated or electrolyzed water, trisodium phosphate, cetylpyridinium chloride, and organic acids (e.g. lactic, acetic acid) (Ricke et al., 2005). In addition, bacteriophage preparations for target bacteria are also included as safe and suitable ingredients used in poultry products (USDA-FSIS, 2012). A *Salmonella*-targeting bacteriophage preparation to be applied to feathers of live poultry prior to slaughter, and bacteriophage preparation of six lytic-phages against *Listeria monocytogenes* can be used on ready-to-eat (RTE) poultry products.

The use of on-line carcass spray washes is commonly found in commercial plants following defeathering and evisceration, which are two possible sources of cross-contamination (Berrang and Bailey, 2009). Food Safety Inspection Service Directive 6420.2 requires a “zero tolerance” standard for visible fecal contamination on poultry carcasses prior to chilling (USDA-FSIS, 2004). Smith et al. (2005) reported an equilibration of contamination (uniform CFU counts) in broiler carcasses during immersion chilling due to cross-contaminants from contaminated to clean carcasses by contact or through the chilling water. It was also suggested the application of antimicrobials during chilling would reduce the cross-contamination by killing bacteria in the water (Smith et al., 2005). Besides the application of antimicrobials in rinses and/or washes or solutions, steam pasteurization or steam vacuum treatments, trimming of contaminated areas, and γ-irradiation or electron beam irradiation have also been developed to sanitize meat and poultry products (Ricke et al., 2005). However, steam pasteurization or steam vacuum is mostly likely to be used on beef carcasses, and application of irradiation may not be viable due to fears as to the use of irradiation on food.

2.3 Bacteriophages

2.3.1 Characteristics and mode of action

Bacteriophages, also well known as phages, are the oldest and most ubiquitous organism on the Earth (Sulakvelidze, 2011). These are naturally occurring viruses that infect bacterial cells. According to Hudson et al. (2005), phages consist of nucleic acid

surrounded by a protein coat. In addition, this author stated that their morphology varies from complex structures, polyhedral head with tail to simple polyhedral. Phages are obligate parasites and their propagation depends on specific hosts (a target genus, serotype or strain) (Soni and Nannapaneni, 2010). In addition, phages cannot infect eukaryotic cell (Sulakvelidze, 2011). This host specificity relies on phage interaction only with a particular set of bacteria that express distinct binding sites or receptors (Joerger, 2003).

Bacteriophages can be divided in two groups differing in their replication cycles. Virulent or lytic phages attach to the bacterial cell through the attachment of tail fibers to specific cell surface receptors, introduce their phage genome into the cell where it is expressed (Hudson et al., 2005; Sulakvelidze, 2011). After DNA injection, phage DNA assume control of the host's biological system stopping the synthesis of the host components, and allowing phage DNA replication and production of capsids within the cell for new phages assembly (Sulakvelidze, 2011). Phage-encoded enzymes destroy host cell wall releasing new phages and killing the host organism (Hudson et al., 2005). Temperate phages integrate their DNA with the host DNA and may transfer integrated fragments of bacterial DNA into another host (Sulakvelidze, 2011). Lytic phages are preferred for phage therapy (Joerger, 2003; Hudson et al., 2005; Sulakvelidze, 2011). According to Sulakvelidze (2011), there are two reason for using lytic phage rather than temperate ones. First, lytic phages are more effective killers against the target host. As for targeting bacteria, they will not represent a hazard for beneficial bacterial flora. Second, lytic phages are safe because they are incapable of transduction or transference

of fragments of host DNA into other bacterial strain or specie, which could give rise to a new virulent bacterium.

2.3.2 Applications in the food industry

Bacteriophage antibacterial activity was early discovered by Ernest Hankin in1896 and Frederick Twort in 1915; however, its therapeutic application for treating human bacterial infections was attributable to Felix d'Herelle in 1919 (Garcia et al., 2008). The interest in bacteriophages as therapeutic agents was displaced in the West around 1940s and 1950s with the arrival of antibiotics (Sulakvelidze, 2011). The widespread use of antibiotics as therapeutic agents in animal production, the early use as growth promotants, and the emergence of antibiotic-resistant organisms have raised the interest in alternative antibacterial approaches such as the re-emergence bacteriophage applications. According to Garcia et al. (2012), phages can be widely applied in: food safety, agriculture, animal veterinary, aquaculture, wastewater treatment, surface disinfection, bacteria detection and environmental remediation.

In order to prevent foodborne diseases, it has been suggested that bacteriophages be used as biocontrol agents in food. This interest stimulates research focused on animal therapy and food safety. Bacteriophages as therapeutic agents have been studied on cattle, sheep, and poultry to treat *Escherichia coli* O157:H7, *Salmonella* and *Campylobacter* (Berchieri et al., 1991; Carrillo et al., 2005; Fiorentin et al., 2005a; Toro et al., 2005; Wagenaar et al., 2005; Sheng et al., 2006; Andreatti Filho et al., 2007; Atterbury et al., 2007; Borie et al., 2008; Callaway et al., 2008; Ricci and Piddock,

2010). Research related to bacteriophage application in several food products also is available and described below.

2.3.2.1 Fish, meat and poultry

Soni and Nannapaneni (2010) researched the reduction of *Listeria monocytogenes* on raw salmon fillets after the application of bacteriophage Listex^{TM} P100. A concentration of 10^8 PFU/g was necessary to yield reductions of bacterial counts of approximately 1.8, 2.5 and 3.5 log CFU/g at initial loads of 2.0, 3.0, and 4.0 log CFU/g, respectively, at 4°C and 22°C (Soni and Nannapaneni, 2010). Similarly, reduction of *Salmonella* on experimentally contaminated fresh channel catfish fillets with *L. monocytogenes* (~4.3 log CFU/g) was observed by using the same phage (7.3 log PFU/g). Reduction on bacterial counts were on the order of 1.4 to 2.0 log at 4°C, 1.7 to 2.1 log at 10°C, and 1.6 to 2.3 log at 22°C (Soni et al., 2010). Bacterial count reductions greater than 1.0 log unit were presented after 30 min exposure compared to 15 min (less than 1.0 log reduction).

Escherichia coli O157:H7 control on beef was evaluated by O'Flynn et al. (2004) using a cocktail of three bacteriophages (e11/2, e4/1c and pp01). Beef pieces were inoculated with 100µl of 10^3 CFU/ml rifampicin-resistant *E. coli* O157:H7 and allowed attach for 1 h, then phage was applied at 10^8 PFU/ml. Seven of the nine contaminated samples treated with phage cocktail presented no sign of *E. coli* O157:H7 and the other two samples showed less than 10 CFU/ml after incubating phage-treated meat samples at 37°C for 1 h (O'Flynn et al., 2004). Additionally, combined antimicrobial effect of nisin

and bacteriophage against *Listeria monocytogenes* was conducted on raw beef; however, no combined phage-nisin action was reported (Dykes and Moorhead, 2002).

Goode et al. (2003) investigated the reduction of *Salmonella* and *Campylobacter* on chicken skin by the application of bacteriophages. Chicken skin samples were contaminated with *S.* Enteritidis and phages were applied at a multiplicity of infection (MOI) of 1, and 100 to 1,000 resulting in less than 1.0 log reduction and up to 2.0 log reduction, respectively. Atterbury et al. (2003) reported a significant reduction of *Campylobacter jejuni* at 4°C for up to 5 days after a titer phage treatment of 10^7 PFU on artificially contaminated chicken skin. *Campylobacter* recovery dropped by 1.1 to 1.2 $\log_{10}$ when samples were previously inoculated with 10^6 CFU inoculum, and 1.1 to 1.3 $\log_{10}$ with 10^4 CFU inoculum compared to their respective controls. Higgins et al. (2005) reported significant reduction of *Salmonella* recovery from broiler carcasses and turkeys when using 10^8 or 10^{10} PFU. Likewise, Bielke et al. (2007) conducted four trials where broiler carcasses were inoculated with *S.* Enteritidis or *S.* Typhimurium, and sprayed with phages (10^9 PFU/carcass). In the four trials, recovery of *Salmonella* Enteritidis was significantly reduced (greater than 70% in two trials), and it was not detected in the other two trials. *Salmonella* Typhimurium was also significantly reduced in two trials (Bielke et al., 2007).

2.3.2.2 Fresh produce

Recently, Pao et al. (2004) reported a 1.5 log reduction in *Salmonella* (serovars Enteritidis, Typhimurium and Montevideo) growth in the soaking water of broccoli

seeds (approximately at 10^2 to 10^3 CFU/g) post-treated with a mixture of two phages (10^8 PFU/ml) at 25°C for 24 h. Studies conducted on fresh-cut honeydew melon for the reduction of *L. monocytogenes* showed that application of phages (by spraying) before contamination could reduce bacterial counts up to 6.8 log units after 7 days at 10°C (Leverentz et al., 2004). Higher concentrations (10^8 PFU/ml) of phage were recommended for more effectiveness. In addition, an improvement in *Listeria* reduction was observed when phage treatment was combined with nisin (bacteriocin). Leverentz et al. (2003) reported bacterial reduction on honeydew melons on the order of 2.0 to 4.6 log units after phage treatment (7days, 10°C); however, greater reductions were obtained when samples where applied with phage and nisin (5.7 log units). Apple slices treated with phage and bacteriocin only showed up to a 2.3 log reduction compared to less than a 0.4 log unit reduction when phage was applied alone. In the case of *Salmonella* control, approximately 3.5 log reduction was reported on honeydew melons stored at 5°C for120 h and 10°C for 48 h, and approximately 2.5 log reduction was obtained at 20°C held for 24 h (Leverentz et al., 2001). *Salmonella* was not significantly reduced on apples at any of these temperatures.

Sharma et al. (2009) investigated the effectiveness of a mixture of three bacteriophages in reducing *E. coli* O157:H7 on fresh-cut lettuce and cantaloupes. Lettuce pieces were inoculated (ca. 3.0 log CFU/cm^2) and treated with phages (ca. 7.0 log PFU/cm^2). Similarly, cut cantaloupe pieces were inoculated (ca. 5.0 log CFU/ml) and treated phages at a concentration of 7.0 log PFU/ml. Lettuce samples treated with phages showed significantly lower counts compared to controls at days 0, 1 and 2 of

storage at 4°C. Cantaloupe pieces presented significant reduction of *E. coli* O157:H7, after 7 days of storage at 4°C. Likewise, sliced cabbage and lettuce leaves inoculated with *L. monocytogenes* (10^3 CFU/g) showed bacterial count reduction greater that 2.0 log after application of phage A511 or P100 (Guenther et al., 2009). Higher doses of phage (10^8 PFU/g) showed to be more effective than lower ones. The reduction of *E. coli* O157:H7 on tomato, spinach and broccoli was examined by Abuladze et al. (2008). In this experiment, a phage cocktail (ECP-100) at three different concentrations (10^8, 10^9, 10^{10} PFU/ml) was applied to experimentally contaminated food samples. Reduction on the bacterial count was observed in a range of 94% to 100% for the food samples.

2.3.2.3 Processed products

Some studies were conducted to evaluate the virulent effect of broad-host-range phages in the reduction of *Salmonella* Typhimurium (Guenther et al., 2012) and *L. monocytogenes* (Guenther et al., 2009; Guenther and Loessner, 2011) in different ready-to-eat (RTE) products. Reduction of *Salmonella* Typhimurium in hot dogs, cooked and sliced turkey breast, mixed seafood, chocolate milk and pasteurized egg yolk was obtained after phage FO1-E2 application (10^8 PFU/g). Results indicated that no viable bacterial cells were present on the food samples after 6 days at 8°C. At 15°C for 6 days, bacterial counts were lowered in a range of 3.0 to 5.0 log on deli meat, chocolate milk, hot dogs and seafood. Egg yolks showed a reduction in approximately 2.0 log after 2 days storage, and then an increase similar to the control samples at day 6 (Guenther et al., 2012).

Phage A511 (10^8 to 10^9 PFU/ml) was tested for reduction of *L. monocytogenes* in soft cheeses. Data showed that reduction greater than 3.0 log for Limburger-type cheese after 22 days of ripening. Lower reductions (2.5 log) were found in Camembert-type cheese after a 21 day ripening period (Guenther and Loessner, 2011). It was concluded that a single higher concentration (10^9 PFU/ml) could be enough for phage treatment during ripening. The same phages A511 and P100 were tested previously in other RTE products. Reductions greater than 2.0 log cycles in *Listeria* counts were reported on previously inoculated samples of chocolate milk, mozzarella cheese brine, hot dogs, and seafood, after 6 days at 6°C when phages were individually applied at 10^8 PFU/g (Guenther et al., 2009).

A combined effect of phage-encoded lysins and nisin for the control of *Staphylococcus aureus* in pasteurized milk was studied by Garcia et al. (2010). The author demonstrated that the lytic effect of the endolysins was dependent on the ionic requirement of the medium (Ca^{++}, Mg^{++} and NaCl). In addition, laboratory tests showed a strong synergistic effect against *S. aureus* when using lysins and nisin together, which was then confirmed to be effective in pasteurized milk for inhibiting the pathogen. Suppression of *Salmonella* Typhimurium DT104 growth in chicken frankfurters contaminated with approximately 300 CFU has also been studied (Whichard et al., 2003). Reductions in the order of 1.8 and 2.1 log units were achieved with two phages (bacteriophage Felix O1 wild-type and a variant) at a 1.9×10^4 PFU/CFU ratio when samples were held at 22°C for 24 h.

2.3.3 Challenges in the use of bacteriophages

Although bacteriophages have been suggested to be an alternative to antibiotics in animal production and processing, this new approach also presents some limitations, described as follows, by Sillankorva et al. (2009). First, specific phages are required for specific strains and specific environmental conditions; an individual phage is not capable of killing all strains of a particular bacterial species. Another issue is the possibility of the development of bacterial mutants resistant to phages as a result of several applications of phage. There is a limitation in the selection of the host for phage production. Target pathogenic strains responsible for real infections need to be used for the production of phage products and cocktails, and non-lysogenic hosts must be selected. Likewise, Garcia et al. (2008) indicated that the phage action showed on laboratory conditions could be greatly reduced when evaluated the same phages on food system. Limited diffusion rates reducing the possibility of phage-host interaction, microbial load which acts as a barrier of specific binding sites required for phage action, temperature and pH are additional limiting factor in the use of bacteriophages as antimicrobials (Garcia et al., 2008).

In addition, some criteria should be considered when selecting phages targeting food pathogens (Mahony et al., 2011): Phages should present a lytic replication pathway which can be confirmed through the genome sequence of phage evaluation prior its application in particular food systems; specific or broad phage host range should be assessed for the target the desired bacteria; ability of the phage to work on specific food

systems should be tested; and efficacy of the phage at the desired temperature should be evaluated.

2.3.4 Regulatory status

Lytic phages have appeared as viable intervention strategy in food safety. Phage and phage mixtures have been developed by companies targeting primarily human and animal infections, food safety, and environmental applications. In the U.S., 21 Code of Federal Regulation (CFR) Part 172.785 includes *Listeria*-specific bacteriophage preparation as an approved additive (FDA-HHS, 2006). In addition, FSIS Directive 7120.1 describes specific products, amount and labeling requirements for bacteriophages for use in meat, poultry and eggs products as food additive (USDA-FSIS, 2012). Companies, such as OmniLytics Inc., Intralytix Inc. and EBI Food Safety, target food safety application of phage-based products. Some of them have obtained regulatory approval in the U.S.

OmniLytics Inc. developed and commercializes AgriPhage^{TM}, a phage cocktail which has received Environmental Protection Agency (EPA) registration for application on produce (Garcia et al. 2012). In addition, the USDA issued two no objection letters to the use of *E. coli* O157:H7 (OmniLytics, 2007) and *Salmonella* (OmniLytics, 2008) bacteriophage preparations on hides of livestock prior to slaughter and feathers of live poultry before processing.

EcoShield^{TM}, a bacteriophage effective against *E. coli* O157:H7 manufactured by Intralytix Inc., obtained regulatory approval from the FDA through a “Food Contact

Notification" (FCN No. 1018) for its use in red meat parts and trim prior to grinding (Goodridge and Bisha, 2011). ListShield™ is another commercial product of Intralytics Inc. that can be applied to RTE meat and poultry products for the control of *L. monocytogenes*. FDA approved it as direct food additive (Sharma and Sharma, 2012). EBI Food Safety, a Netherlands-based company, developed Listex™ P100 bacteriophage product for control of *L. monocytogenes*. At first, the phage-based product obtained the FDA's approval for its use in cheese, and then extended its approval for food in general, including meat and poultry products. The approval granted was as generally recognized as safe (GRAS) (FDA, 2006; FDA, 2007).

As mentioned above, several companies have invested in the production and research of phage-based products and its potential use in the control of foodborne pathogens such as *Salmonella*. This increasing interest in phages as antimicrobials on food has promoted the conduction of further studies focused on the effectiveness of individual or mixtures of phages on target bacteria with minimal risks to human health. Likewise, the participation of regulatory agencies has promoted the guidance on preparation and application of phages as more data becomes available.

3. MATERIALS AND METHODS

3.1 Bacterial strains and preparation of pathogen cocktails

Five wild-type *Salmonella* strains corresponding to the serovars Enteritidis, Typhimurium, Kentucky, Hadar, Heidelberg, and one strain resistant to nalidixic acid (NA), *Salmonella* Typhimurium were used in this study. These strains were isolated from poultry and raw meat. Appropriate antibiotic-resistant isolates were developed from the wild-type strains. All *Salmonella* isolates were maintained on tryptic soy agar (TSA) slants for propagation of pathogenic strains. Slants were kept at 4°C for 3-4 weeks.

Strains were cultured in 10 ml of tryptic soy broth (TSB) and passed twice (37°C, 24 h) to obtain cell concentrations of ca. 10^8 CFU/ml. For the first experiment, two stock cocktail mixtures of antibiotic-resistant strains were prepared by mixing equal volumetric parts of each freshly cultured strain. Cocktail EKT included *Salmonella* Enteritidis, Typhimurium and Kentucky; Cocktail HH contained *S*. Hadar and Heidelberg. Cocktail dilutions were prepared from stock cocktails with 0.1% Peptone Water (PW) targeting 10^4 CFU/g following product inoculation. NA-resistant *Salmonella* Typhimurium was cultured in similar fashion to other *Salmonella* isolates.

3.2 Antibiotic susceptibility testing

Wild-type isolates of *S*. Enteritidis, Typhimurium, Kentucky, Hadar and Heidelberg, were examined with Sensititre™ (Trek™ Diagnostic System Inc.,

Cleveland, OH) for susceptibility to 15 antimicrobial agents as described by Nayak et al. (2007). Sensititre system used the broth microdilution technique, and was interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2010). Susceptibility testing was conducted according to manufacturer instructions with the Gram-negative minimum inhibitory concentration (MIC) plates (CMV1AGNF) utilized by the CDC's National Antimicrobial Resistance Monitoring System.

3.3 Production and evaluation of antibiotic-resistant strains

Bacterial strains resistant to rifampicin (RIF, 200 μg/ml) were developed using a modification of the method of Blackburn and Davies (1994). Wild-type strains were individually inoculated into 10 ml TSB tubes, passed twice, incubating at 35°C for 24 h each pass. Tryptic soy broth containing antibiotic (200 μg antibiotic/ml) bottles containing 90 ml of media were inoculated with each incubated culture tube (10 ml), which then were incubated at 35°C for 24 h. After incubation, RIF-TSA plates were streaked and then incubated at 35°C for 24 h to obtain isolated colonies. Tryptic soy broth and Tryptic soy agar mediums containing rifampicin were prepared by adding 0.2 g RIF (Sigma-Aldrich Co., St Louis, MO) dissolved in 5 ml of N,N-Dimethylformamide (DMF) to 1 L of sterilized media.

The growth curve of each *Salmonella* rifampicin-resistant strain was compared with that of the parent (wild type) strain using an adaptation of the method of Cabrera-Diaz (2007). Each strain was individually cultured in TSB from TSA slants, and incubated twice at 37°C for 18-24 h. After second transfers, dilutions were prepared

using 0.1% PW, and 0.1 ml of the dilution 1:10,000 was transferred to TSB (100 ml) bottles to achieve an initial concentration of 2.0 $\log_{10}$ CFU/ml. The inoculated bottles were incubated at 37°C. Appropriate decimal dilutions in 0.1% PW were prepared and spread on TSA plates at 0, 2, 4, 6, 8, 12, 18, 24 h. At 24 h, each parent strain was also streaked on RIF-TSA plates from incubated culture to confirm that corresponds to pure wild type strains. Parent strains were not expected to grow in antibiotic-containing media. In addition, decimal dilutions from antibiotic-mutant strains were spread plated on RIF-TSA plates. Tryptic soy agar plates containing rifampicin (100 μg/ml) were prepared by adding 0.1 g RIF dissolved in 5ml of DMF into 1 L of sterilized media. All plates were incubated at 37°C and colonies were enumerated after 24 h. This procedure was conducted in triplicate, and growth data was plotted as a function of time (h) and means of *Salmonella* population ($\log_{10}$ CFU/ml).

3.4 Bacteriophage source and application

A mixture of commercially available bacteriophages, Phage-A, Phage-B and Phage-C, active against multiple serovars of *Salmonella* were used in this study. Phage stock had 10^{10} PFU/ml, confirmed independently by a corporate testing laboratory. Phage was applied by spraying with a basic spray gun calibrated to deliver 0.7-1.0 ml of the mixture of bacteriophages per sample (Model #250-2, Badger Air-Brush Co., Franklin Park, IL).

3.5 Bacteriophage infectivity assay

An infectivity assay to evaluate the phage action on individual *Salmonella* strains was conducted by a corporate testing laboratory as described below. Each wild-type and RIF-resistant *Salmonella* strain was grown in Luria Bertani broth (LUB) and incubated at 37°C for 24 h. Overnight bacterial culture (200 µl) was added to sterile LUB top agar at 48°C, and gently vortexed preventing the formation of air bubbles. Soft agar mixture was poured onto a pre-warmed LUB agar plate, evenly distributed by gentle rotation of the plate, and allowed to harden at room temperature. After agar had solidified, 10 µl of serially diluted phage suspension (phage stock 10^{10} PFU/ml) in sterile SM buffer was spotted, incubated at 37°C for 24 h. Each bacteria isolate was individually evaluated with the three bacteriophages (Phage-A, Phage-B, and Phage-C). After incubation, the number of visible plaques was counted. Plaque forming units per milliliter were determined and expressed as $\log_{10}$ PFU/ml.

3.6 Chicken breast tissue samples

Boneless, skinless chicken breasts were obtained from a local commercial poultry processor. Refrigerated chicken breasts were cut into 25 g samples, stored frozen at -15°C (5°F) and thawed at 4°C (39.2°F) for 24 hours prior to each experiment. A total of 84 samples (25 ± 2 g each) were used for each *Salmonella* cocktail for the first experiment. Treatments included a negative control, *Salmonella*-inoculated positive control, *Salmonella* positive treated with phage applied at 10^5, 10^6, 10^7, 10^8, or 10^9 PFU/ml. This experiment was completed with two duplicate identically prepared

samples produced for each treatment/*Salmonella* cocktail/time point (7 treatments x 2 *Salmonella* cocktails [EKT and HH] x 1 storage temperature [4°C (39.2°F)] x 2 samples x 6 time points [0, 15, 30, 60, 120, 360 min] = 168 distinct analytical units).

The second experiment consisted of a total of 64 samples (25 ± 2 g each) distributed as four treatments: a negative control, *Salmonella* positive control, and *Salmonella* positive treated with phage at 10^5 or 10^9 PFU/ml. This experiment was repeated with duplicate identical samples being produced for each treatment/storage temperature/time point (4 treatments x 1 *Salmonella* strain x 2 storage temperatures [4°C (39.2°F) and 25°C (77°F)] x 2 samples x 4 time points [0, 30, 60, 120 min] = 64 distinct analytical units).

3.7 Inoculation and sampling of chicken breast samples

For Experiment 1, refrigerated samples were inoculated by dipping for 5 min in *Salmonella* cocktail dilutions targeting 10^4 CFU/g on meat surfaces. After dipping, samples were allowed to drip for 3 min and attach for 30 min at 4°C. Each sample was then surface treated on both sides with appropriate concentrations of phage: 10^5, 10^6, 10^7, 10^8 and 10^9 PFU/ml. The negative control corresponded to samples dipped in PW and sprayed with RO-deionized water. *Salmonella* positive controls consisted of samples inoculated with *Salmonella* cocktail and sprayed with RO-deionized water. Two duplicate samples were used for each treatment per cocktail. Samples were aseptically placed in a filter stomacher bag (1.63 L capacity) containing 100 ml of PW and stored at

4°C. Sampling times were 0, 15, 30, 60, 120, 360 min post-treatment for enumeration of *Salmonella* colonies.

During Experiment 2, refrigerated samples were inoculated by dipping for 5 min in *Salmonella* dilution targeting 10^4 CFU/g on surfaces. After dipping, samples were let to drip for 3 min and attach for 30 min. Half of the samples were kept at room temperature (25°C) for attachment, whereas the rest was kept at 4°C. Each sample was then surface treated on both sides with appropriate concentrations of phage: 10^5 and 10^9 PFU/ml. The negative control and *Salmonella* positive control followed the procedure described above. Two duplicate samples were used for each treatment/temperature/ time point combination. Samples were aseptically placed in a filter stomacher bag containing 100 ml of PW. Samples resulting from cold attachment were stored at 4°C, whereas samples from room temperature attachment were kept at 25°C. Sampling times were 0, 30, 60, 120 min post-treatment for enumeration of *Salmonella* colonies.

3.8 *Salmonella* spp. enumeration

At each time point, corresponding samples were homogenized at 200 rpm for 1 min in a stomacher in a pouch containing 100 ml PW in addition to sample tissue. Two ml of the homogenate were then concentrated by centrifugation at 13,000 x g for 1 min. Supernatant was discarded and pellets were re-suspended in 2 ml of ice cold PW and centrifuged for a second time (13,000 x g, 1 min). This allowed the separation of the phages from the sample rinsate prior to direct plating. After discarding supernatant and re-suspending pellet in new ice cold PW, serial dilutions were prepared and spread on

Brilliant Green (BG) plates with RIF (100 μg/ml) or NA-BG plates (25 μg/ml NA) as appropriate. Plates were incubated at 37°C for 24-48 h. Colonies of typical *Salmonella* morphology, white to red, opaque colonies surrounded by red zones in the medium, were enumerated on the selective medium.

3.9 Data analysis

Salmonella counts were converted to $\log_{10}$ CFU/ml or g. Growth data ($\log_{10}$ CFU/ml) were plotted as a function of time, and growth parameters such as initial population (N_0), maximum population density (N_{max}), maximum specific growth rate (μmax), lag phase time (t-lag) and doubling time (t-d), were estimated using MicroFit (v.1.0, Institute of Food Research, Norwich, UK). Growth parameters were analyzed with the general lineal model (GLM) procedure of SAS (v.9.3, SAS Institute, Cary, NC) with a significance level of $P < 0.05$. Least squares means were calculated, and if differences ($P<0.05$) were determined by analysis of variance (ANOVA), differences between least squares means were defined using the PDIFF function of SAS. Differences in means for each experiment were calculated by time and treatment, subtracting the log counts obtained at each exposure time (min) from the initial log count obtained at 0 min.

4. RESULTS AND DISCUSSION

4.1 Antibiotic susceptibility testing

Salmonella serovars (Enteritidis, Typhimurium, Kentucky, Heidelberg and Hadar) were selected from poultry origin, based on their prevalence in foodborne illnesses and outbreaks. All these, except Kentucky, were reported to be in the top 20 laboratory-confirmed *Salmonella* serotypes isolated from human sources in 2009 with *S.* Enteritidis (17%) as the most common (CDC, 2011a). This report also included *S.* Kentucky in the top 20 laboratory-confirmed *Salmonella* serotypes from clinical (rank 15) and non-clinical (rank 1) isolated from non-human sources. In the 2008 Surveillance for Foodborne Disease Outbreak Report (CDC, 2011b), *S.* Enteritidis was indicated as the most common (28%) serovar associated with *Salmonella* foodborne outbreaks, followed by *S.* Typhimurium (17%) and *S.* Heidelberg (7%). In addition, the 2010 Retail Meat Report (CDC, 2012b) presented these five isolates among the most common in chicken breast in the following order: Typhimurium (46%), Enteritidis (16%), Heidelberg (12%), Kentucky (12%), and Hadar (12%).

Resistance profiles for wild-type *Salmonella* serovars to antimicrobial agents are shown in the Table 1. Minimum Inhibitory Concentrations (MICs) of antimicrobials were obtained for each isolate, and results were evaluated and reported according to CLSI guidelines (2005). The majority of strains showed susceptibility to antimicrobials from the 8 classes tested: Aminoglycosides, β-lactam/β-lactamase, Penicillins, Cephems, Phenolics, Quinolones, Tetracyclines, Folate Pathway Inhibitors.

Table 1. Resistance profiles for wild-type *Salmonella* isolates.

CLSI class	Antimicrobial Agent	Profile[a]				
		Ent[b]	Had[b]	Heid[b]	Kent[b]	Typh[b]
Aminoglycosides	Amikacin	S	S	S	S	S
	Gentamicin	S	S	S	S	S
	Kanamycin	S	S	S	S	S
	Streptomycin	NI	R[c]	NI	NI	NI
B-lactam/β-lactamase inhibitor combinations	Amoxicillin/ Clavulanic Acid	S	S	S	S	S
Penicillins	Ampicillin	S	S	S	S	S
Cephems	Cefoxitin	S	S	S	S	S
	Ceftiofur[d]	NI	NI	NI	NI	NI
	Ceftriaxone	S	S	S	S	S
Phenolics	Chloramphenicol	S	S	S	S	S
Quinolones	Ciprofloxacin	S	S	S	S	S
	Nalidixic Acid	S	S	S	S	S
Tetracyclines	Tetracycline	S	R	S	S	S
Folate pathway inhibitors	Trimethoprim/ Sulphamethoxazole	S	S	S	S	S
	Sulfisoxazole	S	S	S	S	S

[a]S, susceptible; R, resistant; NI, not interpretable (no interpretations from CLSI).
[b]Ent: *S.* Enteritidis; Had: *S.* Hadar; Heid: *S.* Heidelberg; Kent: *S.* Kentucky; Typh: *S.* Typhimurium.
[c]No CLSI breakpoints; resistance breakpoint used in NARMS is ≥ 64μg/ml.
[d]Third-generation cephalosporin, no described in CLSI guidelines.

For *S.* Hadar, resistance was only shown against Streptomycin (MIC > 64 μg/ml) and Tetracycline (MIC > 32 μg/ml). Results for *S.* Hadar agree with the NARMS 2010 Retail Meat Report (CDC, 2012b), which also reported simultaneous resistance of this serovar to both Streptomycin and Tetracycline in chicken breast. Several studies also

supported the resistance pattern to these two antimicrobial agents (Bokanyi et al., 1990; D'Aoust et al., 1992; Manie et al., 1998; Antunes et al., 2003).

The widespread use of antibiotics in animal disease prevention may contribute to the selection of antibiotic-resistance microorganisms. These resistant organisms are shed in the feces and can be spread from animal to animal and through the environment. CDC (2012a; 2012b) recommends ciprofloxacin, ceftriaxone and trimethoprim-sulfamethoxazole as the first-line antimicrobials for treating salmonellosis. Reports from NARMS (CDC, 2012a) observed antibiotic-resistant isolates not susceptible to nalidixic acid and ceftriaxone. As well as resistance to antibiotics, *S*. Typhimurium (96% within this serovar), *S*. Enteritidis (7%), *S*. Heidelberg (38%), *S*. Kentucky (86%) and *S*. Hadar (100%) were found in strains recovered from retail chicken breast (CDC, 2012b).

4.2 Production and evaluation of antibiotic-resistant strains

Antibiotic resistance was induced for wild-type strains of *S*. Enteritidis, Typhimurium, Hadar, Heidelberg and Kentucky to be used in this study. A high concentration of RIF (200μg/ml) was used to induce antibiotic resistance, which is approximately three times as the maximum acceptable quality control range (16 - 64 μg/ml) for the Gram-negative *Pseudomonas aeruginosa* ATCC® 27853 (CLSI, 2005). MICs for RIF against *Salmonella* are not described in the CLSI guidelines.

Spontaneous RIF-resistant were obtained for each of the five *Salmonella enterica* serovars, and their growth characteristics were evaluated and compared to validate the use of these resistant strains in further experiments. It was expected that the behavior of

antibiotic resistant mutant of each serotype did not differ from the wild-type strains as had been demonstrated in early studies (Kim et al., 1995; Castro-Rosas et al., 2010). *Salmonella* Typhimurium (ATCC 6539) and its RIF-resistant strain presented similar growth pattern in TSB, showing consistence bacterial counts on TSA and on RIF-TSA plates (Kim et al., 2005). Likewise, *Salmonella* (three serovars Typhimurium [ATCC 14028; J1; and GA1], one Typhi, one Montevideo and one Gaminara) RIF^+ and *E. coli* (ATCC 25922, ATCC 35218, and ATCC 10536) RIF^+ did not differ from their parent strains (Castro-Rosas et al., 2010). Even though the concentration of antibiotic in the present research was higher (200 μg/ml) than the one utilized in these two studies (100 μg/ml), there were not significant differences among growth parameters of parent and RIF-resistant strains.

After comparing growth parameters between RIF-resistant *Salmonella* strains and their nonresistant serotypes, no difference ($P > 0.05$) was found for each parameter by each serotype (Table 2). In addition, there was no significant difference when comparing the growth behavior among all the strains. These results confirmed that resistance to the antibiotic RIF at a level of 200 μg/ml did not induce significant physiological changes in *Salmonella* isolates as determined by rate of growth of parent and RIF-resistant mutants for each serotype in nutritious, non-selective medium. The development of resistance on these strains may be due to two factors, the presence of resistance genes and the selective pressure by the use of antibiotics (Levy, 2002; Levy and Marshall, 2004).

Table 2. Growth parameters for wild-type and RIF-resistant *Salmonella* isolates in tryptic soy broth at 37°C

	Mean[a] ± SD[b]				
Isolates	N_0 (log_{10} CFU/ml)	Nmax (log_{10} CFU/ml)	μmax (h^{-1})	t-lag (h)	t-d (h)
S. Enteritidis – Parent	2.3 ± 0.2	9.0 ± 0.0	2.0 ± 0.1	1.6 ± 0.4	0.34 ± 0.02
S. Enteritidis – RIF	2.3 ± 0.2	9.0 ± 0.1	2.0 ± 0.1	1.6 ± 0.3	0.34 ± 0.02
S. Hadar – Parent	2.3 ± 0.3	9.1 ± 0.1	2.1 ± 0.0	1.7 ± 0.5	0.33 ± 0.01
S. Hadar – RIF	2.2 ± 0.2	9.1 ± 0.1	2.1 ± 0.0	1.6 ± 0.4	0.34 ± 0.00
S. Heidelberg – Parent	2.2 ± 0.1	9.1 ± 0.0	2.1 ± 0.1	1.5 ± 0.3	0.34 ± 0.02
S. Heidelberg – RIF	2.2 ± 0.0	9.0 ± 0.1	2.2 ± 0.1	1.6 ± 0.1	0.32 ± 0.01
S. Kentucky – Parent	2.2 ± 0.4	8.9 ± 0.2	2.1 ± 0.1	1.9 ± 0.7	0.33 ± 0.02
S. Kentucky – RIF	2.1 ± 0.3	9.0 ± 0.1	1.9 ± 0.0	1.4 ± 0.2	0.37 ± 0.01
S. Typhimurium – Parent	2.3 ± 0.1	9.1 ± 0.1	2.1 ± 0.2	1.6 ± 0.4	0.32 ± 0.03
S. Typhimurium – RIF	2.2 ± 0.3	9.0 ± 0.1	2.1 ± 0.1	1.6 ± 0.7	0.34 ± 0.02

N_0 = initial bacterial cell density; N_{max} = final bacterial cell density; μmax = maximum specific growth rate; t-lag = lag time; t-d = doubling time

[a]Mean values were obtained from three independent replicates

[b]Standard deviation

Means in the same column with the different letters are different ($P < 0.05$)

Since *S*. Enteritidis, *S*. Typhimurium, *S*. Kentucky, *S*. Heidelberg and *S*. Hadar resistant strains multiplied at the same rate, their combined use as a cocktail/mixture was applied during the present study. Available growth data was fitted per replicate using MicroFit software, verifying its correspondent growth parameter as it presented similar growth models to the other replicates. In addition, a growth of approximately 7.0 $\log_{10}$ CFU/ml ($N_{max} - N_{0)}$ was achieved among all the strains within 24 hours. This finding confirmed the ability of these organisms to obtain predictable numbers following overnight incubation and medium conditions.

4.3 Bacteriophage infectivity assay

An important advantage on the application of bacteriophages as antimicrobial agents is their high host specificity. Phages interact only with specific sets (genus, serotype, strain) of bacteria that express specific binding sites or receptors (Joerger, 2003). Among these receptors, outer-membrane transport proteins, lipopolysaccharide, carbohydrates, flagella and pili can be found (Hudson et al., 2005).

Lytic spectra and $\log_{10}$ PFU/ml for the three phages, Phage-A, Phage-B and Phage-C, are presented in Table 3. Phage-A showed high infectivity for parents and resistant mutants of the five serotypes with high phage counts ($\log_{10}$ PFU/ml), except for RIF- resistant *Salmonella* Kentucky which presented log values in the order of 3.0. Phage-B showed to be effective against *S*. Hadar and *S*. Kentucky whereas Phage-C only lysed and replicated in *S*. Enteritidis. High phage values in the range of 6.3 to 8.1 $\log_{10}$ PFU/ml may indicate the ability of the bacteriophages to replicate in these specific

Salmonella strains. The different behavior among the three phages over the five wild-type *Salmonella* strains (wild-type and resistant) confirmed that there is no known phage that has lytic action for all *Salmonella* serovars as stated by Joerger (2003). These observations were also supported by Sklar and Joerger (2001), who had shown that none of the six phages tested in their study produced plaques on more of the half of the thirteen serotypes tested, including the serotypes Enteritidis, Typhimurium, Hadar, and Heidelberg. Therefore, it was proposed the application of the three bacteriophages as mixture on the following experiments to increase the infective action over the *Salmonella* cocktails to be tested.

Table 3. Lytic spectra and $\log_{10}$ PFU/ml of *Salmonella* bacteriophages determined on 10 *Salmonella* isolates (five wild-type and five RIF-resistant).

	Lysis by bacteriophage[a] and $\log_{10}$ PFU/ml[b]					
Isolates	Phage-A		Phage-B		Phage-C	
S. Enteritidis – Parent	+	8.0	-	ND	+	7.5
S. Enteritidis – RIF	+	8.0	-	ND	+	7.7
S. Hadar – Parent	+	8.0	+	6.3	-	ND
S. Hadar – RIF	+	8.1	+	7.0	-	ND
S. Heidelberg – Parent	+	6.7	-	ND	-	ND
S. Heidelberg – RIF	+	7.0	-	ND	-	ND
S. Kentucky – Parent	+	6.7	+	6.7	-	ND
S. Kentucky – RIF	+	3.0	+	7.6	-	ND
S. Typhimurium – Parent	+	7.8	-	ND	-	ND
S. Typhimurium – RIF	+	7.6	-	ND	-	ND

[a] +, lysis; -, no lysis
[b]ND, not detected

4.4 Effect of phages mixture in reducing *Salmonella* spp.

4.4.1 Experiment 1

Experiment 1 was conducted to determine the load reduction resulting from the application of various levels of a mixture of three bacteriophages on boneless skinless chicken breast inoculated with high levels (10^5, 10^6, 10^7, 10^8 and 10^9 PFU/ml) of *Salmonella* cocktail Enteritidis, Kentucky and Typhimurium (EKT) or Hadar and Heidelberg (HH) at 4°C (39.2°F) up to 360 min. This temperature was chosen to replicate conditions in poultry processing facilities for the application of bacteriophages at the exit of chiller. Raw boneless skinless chicken breast samples used as a control in this experiment yielded negative results for *Salmonella*. In addition, *Salmonella* positive control samples were confirmed to target approximately 4.0 $\log_{10}$ CFU/g on both experiments.

Difference in means for samples contaminated with cocktail EKT and HH are shown in Table 4 and Table 5, respectively. Differences in means for cocktail EKT ranged from 0.1 to 0.7 $\log_{10}$ CFU/g. High differences (0.6 and 0.7 $\log_{10}$ CFU/g) were found after application of phages at a concentration of 10^9 PFU/ml for 120 min, and 10^8 PFU/ml for 30 min, respectively. Applications with 10^5 PFU/ml at 30, 60 and 360 min, and 10^7 PFU/ml at 30 min or greater exposure time were not effective as indicated by no differences. As well, samples treated with 10^6 PFU/ml were not effective at any exposure time. For cocktail HH, differences in means ranged from 0.1 to 0.4 $\log_{10}$ CFU/g with maximum differences (0.4 $\log_{10}$) with concentration 10^7 PFU/ml for 15 min, and 10^9 PFU/ml for 120 min exposure. Primarily, no reductions were found on samples treated

with 10^5 PFU/ml at 120 min or greater exposure time, and 10^8 PFU/ml up to 360 min, except the 60 min time point.

Low differences in means (less than 1.0 $\log_{10}$ reduction) were obtained on Experiment 1 for both cocktails. These observations may be the result of the effect of dosage, temperature and time applied during the study. Results showed that high phage titer was more effective in reducing *Salmonella* counts on both cocktail EKT (10^8 - 10^9 PFU/ml) and cocktail HH (10^7 and 10^9 PFU/ml). These results agree with previous studies conducted on several food products: 10^7 PFU, Atterbury et al. (2003); ca. 7.0 log PFU/cm^2, Sharma et al. (2009); 7.3 log PFU/ml, Soni et al. (2010); 10^8 PFU/g, Guenther et al. (2009), Soni and Nannapaneni (2010), Guenther et al. (2012); 10^9 PFU/carcass, Bielke et al. (2007); 10^9 PFU/ml, Guenther and Loessner (2011); 10^8 PFU/ml, Leverentz et al. (2004), O'Flynn et al. (2004), Pao et al. (2004); 10^8 to 10^{10} PFU/ml, Higgins et al. (2005), Abuladze et al. (2008). However, it is important to note that results from these studies showed reduction equal or greater than 1.5 log cycle, which is higher than the $\log_{10}$ reductions presented on this research.

Therefore, the results in the present study could be due to several factors. In this study it was assumed that phages were completely attached (100%) to the samples, but it is possible that some losses occurred during phage application (spraying) or that the ratio of PFU/CFU was not sufficient which could affect the results. As suggested by Bigwood et al. (2008), a high concentration of phages which exceeds their host density may result in greater reductions as a greater number of host cells become infected. In addition,

Table 4. Difference in means for chicken breast experimentally inoculated with *Salmonella* cocktail EKT after exposure to a mixture of bacteriophages for 15, 30, 60, 120 and 360 min stored at 4°C

	Difference in Means (log_{10} CFU/g)				
	Exposure Time (min)				
Treatment	15	30	60	120	360
Cocktail EKT + 10^5 PFU/ml	0.2	-0.2 [a]	0.0 [a]	0.1	-0.1 [a]
Cocktail EKT + 10^6 PFU/ml	-0.1 [a]	-0.1 [a]	-0.1 [a]	0.0 [a]	-0.3 [a]
Cocktail EKT + 10^7 PFU/ml	0.2	-0.1 [a]	-0.4 [a]	-0.3 [a]	-0.4 [a]
Cocktail EKT + 10^8 PFU/ml	0.1	0.7	0.1	-0.2 [a]	0.2
Cocktail EKT + 10^9 PFU/ml	0.3	0.3	0.3	0.6	0.1

[a] No reduction.

Table 5. Difference in means for chicken breast experimentally inoculated with *Salmonella* cocktail HH after exposure to a mixture of bacteriophages for 15, 30, 60, 120 and 360 min stored at 4°C

	Difference in Means (log_{10} CFU/g)				
	Exposure Time (min)				
Treatment	15	30	60	120	360
Cocktail HH + 10^5 PFU/ml	0.1	0.1	0.1	-0.1 [a]	-0.3 [a]
Cocktail HH + 10^6 PFU/ml	0.1	0.2	0.1	0.1	-0.1 [a]
Cocktail HH + 10^7 PFU/ml	0.4	0.2	0.2	0.2	0.1
Cocktail HH + 10^8 PFU/ml	0.0 [a]	-0.2 [a]	0.1	-0.1 [a]	-0.1 [a]
Cocktail HH + 10^9 PFU/ml	0.2	0.1	0.1	0.4	0.1

[a] No reduction.

refrigeration temperatures (4°C, 39.2°F) may influence *Salmonella* and phage behavior. At stated by D'Aoust and Maurer (2007), *Salmonella* spp. optimally grow at 37°C, and only a few variants are able to grow at extreme temperatures. Therefore, growth at 4°C (39.2°F) may be restricted for this pathogen as was reflected on the results obtained in this study. According to Gill (2010), the ability of a phage to replicate on a mesophilic host in a typical refrigerated food may be limited.

In addition, samples were inoculated with *Salmonella* and treated with phages, then exposed to the phage up to 360 min. At the processing plants, carcasses go from the chiller to further processing or packaging within this period of time. Longer exposure time (8-12 days) were proposed for studies on the application of phages to food at 10°C or 4°C (Soni and Nannapaneni, 2010; Soni et al., 2010). Therefore, longer time exposure (> 24 hours) might be required to obtain significant reduction of bacterial loads. In contrast, one study has indicated mostly significant log reductions (1.1 log at 3 h, 1.8 log at 6 h, 2.0 log at 24h) in *Salmonella* Typhimurium phage P7 (high MOI, 10^4) after phage application to raw meat within 24 h at 5°C with a high host density (10^4 cells/cm^2), whereas no significant differences were found at the same temperature, time points but with low host density (< 10^2 cells/cm^2) (Bigwood et al., 2008). This author also reported higher reduction after 8 days incubation at 5°C. On other hand, the entrapment of bacteria on the meat surface after inoculation by immersion of chicken samples on cocktail solution could affect the action of phages as antimicrobial. As stated by Lillard (1988) the entrapment of bacteria in crevices on tissue surfaces may have a barrier effect against antimicrobials. It is possible that the application of phages by spraying in the

concentrations tested was not as effective as could be through immersion because immersion may cause changes in the micro-topography of the tissue surface, with the expansion of connective tissue within muscles (Thomas and McMeekin, 1981). In this way, phages would be able to reach bacteria already entrapped in muscle surface.

4.4.1 Experiment 2

The purpose of Experiment 2 was to test the efficacy of two levels of bacteriophages (10^5 and 10^9 PFU/ml) on boneless skinless chicken breast experimentally contaminated with a host strain *Salmonella* Typhimurium resistant to nalidixic acid (NA) at two different temperatures, 25°C (77F°) and 4°C (39.2°F) for 0, 30, 60 and 120 min exposure at each temperature. Results are shown in Table 6. Differences in means for NA-resistant *Salmonella* Typhimurium ranged from 0.2 to 0.9 $\log_{10}$ CFU/g at 25°C (77°F), and 0.1 to 0.4 $\log_{10}$ PFU/ml at 4°C (39.2°F). Application of higher phage concentration (10^9 PFU/ml) at room temperature (25°C, 77°F) showed to be the most effective treatment with NA-resistant *S.* Typhimurium compared to samples subjected to refrigeration temperatures. As in the first experiment, phage concentration, temperature, time and bacterial attachment to chicken meat surfaces were factors that may have negatively affected the action of the phages during the experiment. Unlike the first experiment, the effect of room temperature was evaluated for a high (10^9 PFU/ml) and low (10^5 PFU/ml) phage concentration. Room temperature favored the action of the phage at 120 min exposure time compared to refrigeration temperatures (4ºC). As stated previously, refrigeration temperatures were not optimal growth temperature for this host,

as *Salmonella* optimum growth temperature is at 37°C (D'Aoust and Maurer (2007). In respect to phage activity, Hudson et al. (2005) has stated that replication of phages is less effective at approximately 20°C, below the optimum growth temperature for *E. coli* but 10°C above its minimum temperature for growing. This probably may be also true for other enteric bacteria like *Salmonella*, and could explain these results.

Table 6. Difference in means for chicken breast experimentally inoculated with nalidixic acid-resistant *Salmonella* Typhimurium after exposure to a mixture of bacteriophages at 25°C and 4°C for 30, 60 and 120 min

	Difference in Means (log_{10} CFU/g)					
	25°C			4°C		
	Exposure Time (min)			Exposure Time (min)		
Treatment	30	60	120	30	60	120
NA-ST[b] + 10^5 PFU/ml	0.2	0.2	-0.2[a]	0.0	0.1	0.2
NA-ST[b] + 10^9 PFU/ml	0.6	0.5	0.9	0.2	0.4	0.0

[a] No reduction.
[b] Nalidixic acid-resistant *Salmonella* Typhimurium.

5. CONCLUSIONS

This study has shown that application of a mixture of three commercially available bacteriophages can reduce RIF-resistant *Salmonella* spp. counts on experimentally contaminated boneless skinless chicken breasts up to 0.9 log CFU/g. High concentrations of phages, in the order of 10^7 to 10^9 PFU/ml, resulted in more effective phage action as determined by *Salmonella* load reduction. Room temperature conditions (25°C, 39.2°F) favored higher log differences on means of NA-resistant *Salmonella* Typhimurium at 120 min. In addition, time and bacterial attachment on meat surfaces may also affect phage efficacy on chicken breast samples.

Further studies are needed on the effect of phage dosage, greater than 10^9 PFU/ml, on efficacy of the mixture of these three bacteriophages at various time points at both room temperature and refrigerated conditions. This may allow determination of potential effectiveness on processing steps such as picking and post-chilling. Additionally, research focused on increased exposure times may be conducted. Research should also include a combined application of these three bacteriophages with other components such as a bacteriocin-like nisin (membrane permeabilizer) which has been effective against *L. monocytogenes* on melon slices but may require the addition of chelators (e.g. EDTA) to enhance its activity against *Salmonella;* phage-encoded enzymes which lyse bacterial cell walls; and the addition of $CaCl_2$ or divalent cations to the medium to promote phage adherence to target bacteria. These components may be investigated as alternatives to enhance phage action on the present model system.

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